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Fluorescent Probes in the Development of New Analytical Methods for Detection of Water Pollution

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by

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27 Abstract

The application of fluorescent probe-enzyme systems to detection of water pollution by insecticides was studied to determine the scientific and practical feasibility of this new concept. Many suitable fluorescent probe molecules can be synthesized. In combination with serum cholinesterase enzyme, these fluorescent probe-enzyme systems give a significant spectroscopic response in the presence of insecticides. A total of 18 new candidate fluorescent probe molecules were synthesized. Of those tested, 4 are useful probe molecules.

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ABSTRACT

The application of fluorescent probe-enzyme systems to detection of water pollution by insecticides represents a new concept. This research was initiated to determine the scientific and practical feasibility of this new concept.

In this first year of research we have proven that many suitable fluorescent probe molecules can be synthesized. We have also shown that in combination with serum cholinesterase enzyme, these fluorescent probe-enzyme systems give a significant spectroscopic response in the presence of insecticides. Thus, in this first year we have conclusive proof of the scientific feasibility of this new methodology. A total of eighteen new candidate fluorescent probe molecules have been synthesised. Of those tested, four are useful probe molecules.

In the second year of this research we propose to develop our probe-enzyme systems into practical analytical tools. This will be done by (1) synthesis of optimum probe molecules (2) development of optimum enzyme systems (3) study of optimum fluorescence spectroscopic techniques.

I. Objectives

The ultimate objective of this research is to develop a new, highly sensitive, fluorescence spectroscopic detection system for water pollution by insecticides. To accomplish this objective we have developed a research program on fluorescent probe-enzyme systems. Such a system has the critical characteristic of spectral response when any foreign molecule competes for the active site of the enzyme. When the competing, foreign molecule is an insecticide, then the change in fluorescence of the probe-enzyme complex can measure the amount of insecticide pollution in the water sample. The specificity of this type of fluorescence detection system would be a function of the specificity of the enzyme component of the fluorescent probe-enzyme complex. The sensitivity of the system would be a function of the quantum yield and equilibrium constant of the fluorescent probe-enzyme complex.

The development of a suitable fluorescent probe-enzyme complex involves the design and synthesis of candidate probe molecules. These fluorescent probes must be shown to be reversible (equilibrium) inhibitors of the active site of the enzyme involved. When a candidate molecule is found to be both a fluorescent probe and a reversible inhibitor it has a unique importance. Such a molecule and its analogues and homologues are the fundamental basis of this research. They are the fundamental tools for a fluorescence spectroscopic detection system, specific for insecticide pollution.

II. Summary of the Research

A. Fundamentals

A fluorescent probe is defined as a fluorescent molecule whose spectral responses (emission, excitation and quantum yield) are a function of environment. In this research we make use of this rather unusual characteristic by synthesis of designed fluorescent probe molecules which are specific for the active-site of an enzyme and which are in equilibrium with it. Such a fluorescent probe-enzyme complex will have a unique wavelength of emission, different from the emission wavelength of the free probe molecule in water. If the fluorescence quantum yield of the probe-enzyme complex is favorable, and if the equilibrium constant of the complex is also favorable, then such a system can be an elegant, sensitive and specific spectroscopic detection system for insecticide pollution in water.

B. Synthesis program

A total of eighteen new candidate flucrescent probe molecules have been synthesized during this period. On the basis of our prior research we have given priority and emphasis to molecules which contain the 5-dimethylaminonaphthalene-1-sulfonyl moiety. This dansyl moiety is readily synthesized into a variety of molecules and has many favorable fluorescence characteristics. We also have a considerable background on its spectral response and quantum yield characteristics. In addition, we have also studied the sulfonamido derivatives of this grouping to a very considerable extent and have an extensive background on their biological reaction characteristics.

Of the eighteen candidates from the synthesis program, a total of four have been shown to react as fluorescent probes with horse serum cholinesterase and thus to be of specific importance to this research program. These four and five others were shown to be ineffective with bovine erythrocyte acetylcholinesterase. We now believe that the extreme specificity of the acetylcholinesterase may not be a desireable factor and at least for the present we plan to emphasize study of probe-enzyme complexes with serum cholinesterase. A few of the compounds which we synthesized were found to be photolytically unstable and these molecules and their analogues are being removed from the research program. The synthesis program is mainly concerned with the synthesis of carboxylic esters and phosphonate esters containing the dansyl moiety, and with the synthesis of a series of fluorescent sulfonamides.

B. Spectroscopic Program

Our spectroscopic research is carried out with a G. K. Turner Spectro 210, absolute spectrofluorometer. In November 1969, the first successful fluorescent probe-enzyme system was found. At that time, we found a probe molecule which was a reversible inhibitor of horse serum cholinesterase. Subsequently we proved by pH stat studies that the molecule was a competitive inhibitor of the enzyme and thus, in equilibrium with it under conditions favorable to our objectives. Preliminary data were presented in a paper at the Chicago meeting of the Entomological Society of American in December 1969. Subsequently, three additional products of the synthesis program were found to act as fluorescent probes with horse serum cholinesterase.

Our present research is concerned with the development of additional and complete data on the spectroscopic response of the fluorescent probe-enzyme complexes which we have developed. In addition, we also study the kinetics of their reaction with the enzyme in the presence of acetylcholine. The objective of such research is to prove that the probe is, in fact, a competitive inhibitor of the active-site and to obtain complete data as to its characteristics with the enzyme. Finally, we plan to determine the stability of the probe-enzyme complex to light and to other possible degradation.

The importance of the spectroscopic data lies in the fact that until such research is available in depth, no valid estimates of the possible sensitivity and ultimate usefulness of the probe-enzyme complex can be made. This type of research will constitute a major portion of the program for the next year. The crux of the research to be done in the future is to find probe molecules with optimum characteristics and to determine optimum detection parameters. We believe that the program to date has been highly productive of significant new knowledge and important results. It is on, or ahead of schedule.

C. Publications

Two publications pertinent to this research have been published and a third is in press. These are:

1. Himel, C. M. and R. T. Mayer (1970) "5-dimethylaminonaphth - lene-1-sulfonic acid (Dans Acid) as standard for quantum yield of fluorescence". Anal. Chem. 42 130-2.

Many of the compounds synthesized in the synthesis portion of this research contain the dansyl moiety. It is therefore important that a related molecule be available for use as a quantum yield standard. In addition, methodology for the determination of quantum yields is an important part of this research program.

2. Himel, C. M. and R. T. Mayer (1970) "Applications of fluorescence spectroscopy to fundamental problems in insect biochemistry" J. Georgia Entomol. Soc. 5 (1) 31-8.

The importance of this paper lies in the fact that it is the first publication of the applicability of a secondary sulfonamide as a fluorescent probe for the active site of a cholinesterase enzyme. The data in this paper confirm the feasibility of the present research program.

3. Whidby, J. F., D. E. Leyden, C. M. Himel and R. T. Mayer. (1970) "An NMR study of the protolysis kinetics of sulfonamides" J. Am. Chem. Soc. In Press.

This paper reports the first data on the acid and base catalysis of the protolysis kinetics of sulfonamides. NMR spectroscopy was used in this study which was carried out jointly with Professor Leyden in the chemistry department. The data are important in that they constitute a rationale for the biological activity of sulfonamides.

Copies of the publications and a draft copy of the article in press are attached to this report as an appendix.

III. Discussion

Major application of fluorescence spectroscopy to biochemical research is of relatively recent origin. It was not until about 1956 that the ultra-violet fluorescence of proteins was proven to be related to their tryptophan content. In our laboratories we have been particularly interested in the synthesis of new fluorescent molecules which react with the active site of enzyme systems. One of the limitations of fluorescence spectroscopy in the study of biological problems lies in the fact that only a minority of organic molecules are fluorescent. We circumvent this limitation by the design and synthesis of molecules which are fluorescent and which are biologically active. For the research being carried out in this grant, we are particularly interested in fluorescent molecules which are:

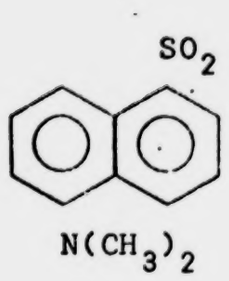
1. Reversible (equilibrium) inhibitors of the active site of cholinesterase enzymes.
2. Competitive inhibitors of the active site.
3. Fluorescent probes for the active site.

Fluorescent probes are defined as fluorescent molecules whose spectral characteristics (fluorescence excitation, emission and quantum yield) are affected by the environment of the probe molecule. When this is the case, the fluorescent probe molecule will have an emission wavelength which is characteristic of the environment of the active site of the enzyme and which will be different from that of the free probe molecule in water. An optimum fluorescent probe for our purposes would have the following characteristics:

1. Low quantum yield in water solution
2. High quantum yield in non-polar solvents
3. A significant hypsochromic shift (to shorter wavelengths) in the emission of the probe-enzyme complex.
4. Photolytic stability
5. A significant degree of inhibition of the enzyme active-site

The probe molecules referred to in this report react at the molecular level. In the process of becoming coincident with the envirnoment of the active site of the enzyme, the fluorescent probe molecule responds to that environment by a shift in its spectral properties. That shift is the effect which we strive to find in our study of candidate probe molecules. When such a shift is observed, we are concerned with the determination of the intensity of the spectroscopic response and its characteristics under conditions of competition for the active site by insecticide molecules.

At the start of this research program, we planned to synthesize candidate probe molecules which contained the 5-dimethylaminonaphthalene-1-sulfonyl moiety (dansyl moiety) as indicated in (1) below: In (1) we indicate the dansyl moiety as part of a secondary sulfonamide prepared from dansyl chloride and N, N dimethyl ethylene diamine.

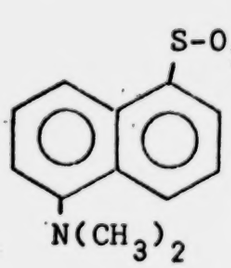


SO_2 ----- $N(H)-CH_2-CH_2-N(CH_3)_2$ Compound 1

--- $N(H)-CH_2-CH_2-CH_2-N(CH_3)_2$ Compound 2

$N(CH_3)_2$ --- $N(H)-CH_2-CH_2-CH_2-CH_2-NH_2$ Compound 3

Compound 1, along with its quaternary derivative is one of the more promising flourescent probe molecules developed in this research. We have also prepared the propyl homologue and the butyl analogue. The quaternary derivative of (2) is shown below as compound (4).



$S-O_2-N(H)-CH_2CH_2-CH_2\overset{+}{N}(CH_3)_3$

Compound 4

$N(CH_3)_2$

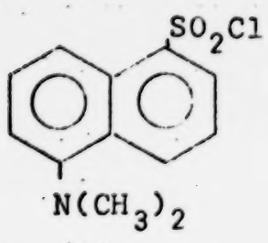
Data from pH stat studies indicate that compounds 1 and 2 are competitive inhibitors of the active site of horse serum cholinesterase, but are not inhibitors of the active site of bovine erythrocyte cholinesterase. For this reason, at the present time we believe that the most promising enzyme system for our studies will be found in the use of aliesterases such as serum cholinesterases. We also plan to study the effect of lipase enzyme systems. Complete study of the spectroscopic response of each probe-enzyme system will involve a thorough study of (1) the

equilibrium constant for the probe as an inhibitor of the enzyme active site (2) proof that the probe in a competitive inhibitor of the active site (3) complete fluorescence emission, excitation and quantum yield data for the probe-enzyme complex and for the free probe. (4) Complete photolytic stability data on the probe-enzyme complex. For these reasons, each candidate probe system studied will involve a considerable amount of research time and effort. As this research progresses we plan to vary the synthesis program to produce candidate probe molecules with optimum characteristics. Such optimum characteristics will be predicted from the data on each probe molecule studied.

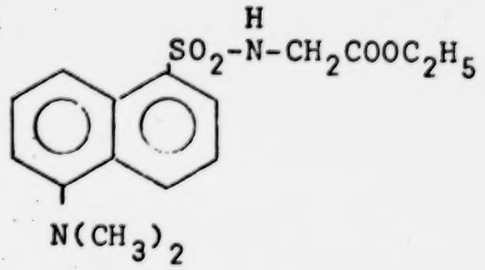
IV. Experimental

A. Synthesis

5-Dimethylaminonaphthalene-1-sulfonyl chloride (5) is readily available and is an effective group for synthesis reactions. The reaction product with glycine is also available commercially and is of possible importance in this research (6)



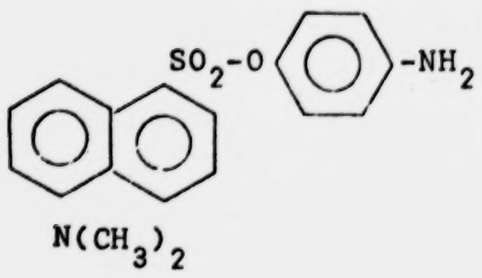
(5)



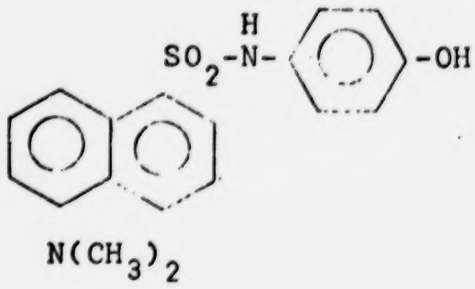
(6)

When dansyl chloride (5) is reacted with a bifunctional molecule such as p-aminophenol, it is possible to produce the sulfonate from reaction with the hydroxyl or the sulfonamide from reaction

with the amino group. We have been able to work out synthetic procedures for specific synthesis of either of these products (7) and (8). Compounds (7) and (8) are used as model compounds for further synthesis. Their homologues from N-methyl p-aminophenol have also been produced.

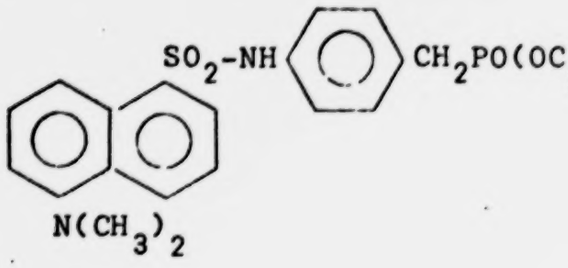


(7)

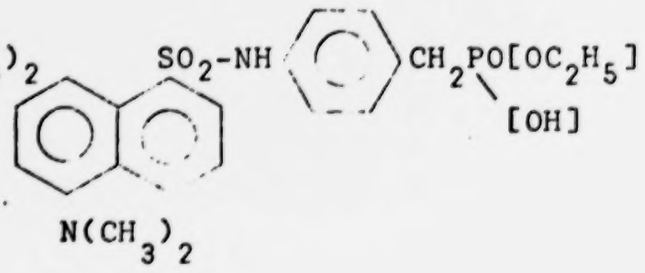


(8)

The phosphonate ester (9) and its mono acid product (10) are of potential importance as probe systems. The former is readily prepared but the latter requires specific conditions. It is produced by controlled hydrolysis of (9).



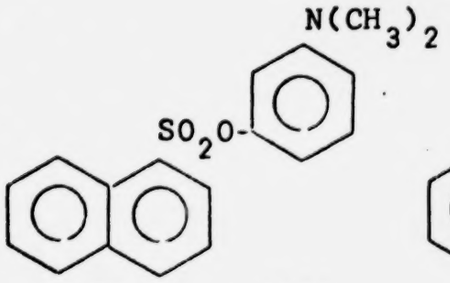
(9)



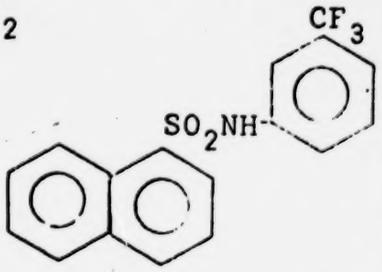
(10)

It is possible that some of the <u>meta</u> substituted systems such as indicated in (11) (12) and (13) could be analogues of many carbamate-type insecticides. This was the rationale underlying

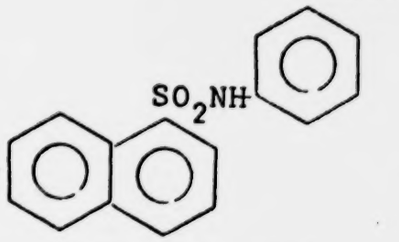
their synthesis. Compound (11) was subsequently shown to be photolytically unstable and was removed from further consideration. Compound (12) is unusually stable and has shown remarkable spectroscopic characteristics. Its ultimate importance is not known at this time.



(11)

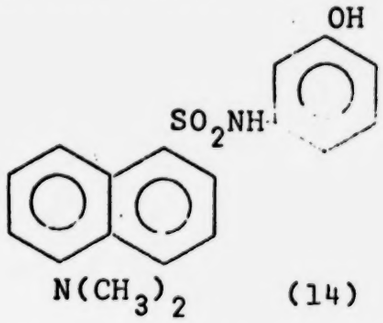


(12)



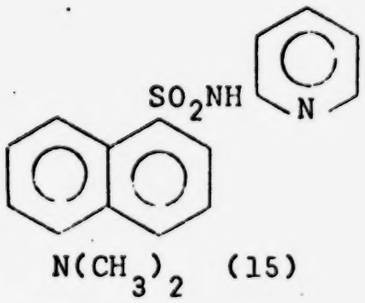
(13)

The <u>meta</u> homologue of (8) was prepared as compound (14) by the same selective synthesis used in (8).

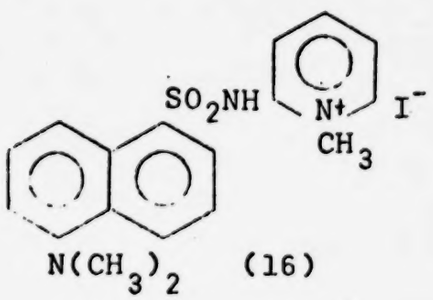


(14)

Included in the synthesis program is a series of pyridine derivatives some of which are shown as compound (15) and (16). In this case, (16) is the quaternary derivative of (15).



(15)



(16)

The rationale for the extensive use of the 5-dimethylaminonphthalene moiety in these syntheses is a part of our prior research and the data published by Whidby, Leyden, Himel and Mayer (1970). An effective probe system requires fluorescence emission in the range of 5,000 to 5,500 A. The synthesis program for the coming year will be concerned with other possible fluorescent moieties, including acridines and anthracene derivatives.

B. Spectroscopy

We have, at this time, standardized on two enzyme systems -- (1) bovine erythrocyte acetylcholinesterase and (2) horse serum cholinesterase. The latter appears to be more adaptable to our needs and we plan to use it to a major extent. Later research will be done to study the effect of lipase enzymes. Since an optimum probe-enzyme system requires a hypsochromic shift of the emission of the probe-enzyme complex, the polarity of the active site of horse serum cholinesterase appears to be close to optimum. This will, however, be checked with a study of the possible utility of lipase enzymes.

Fluorescence emission spectra for compound (2) are given in Figure 1. In Figure 1, curve A represents the emission of the probe-enzyme complex and the heart of a possible spectroscopic detection system. Figure 1 does not represent optimum conditions, hence we consider these data to be highly important and very encouraging.

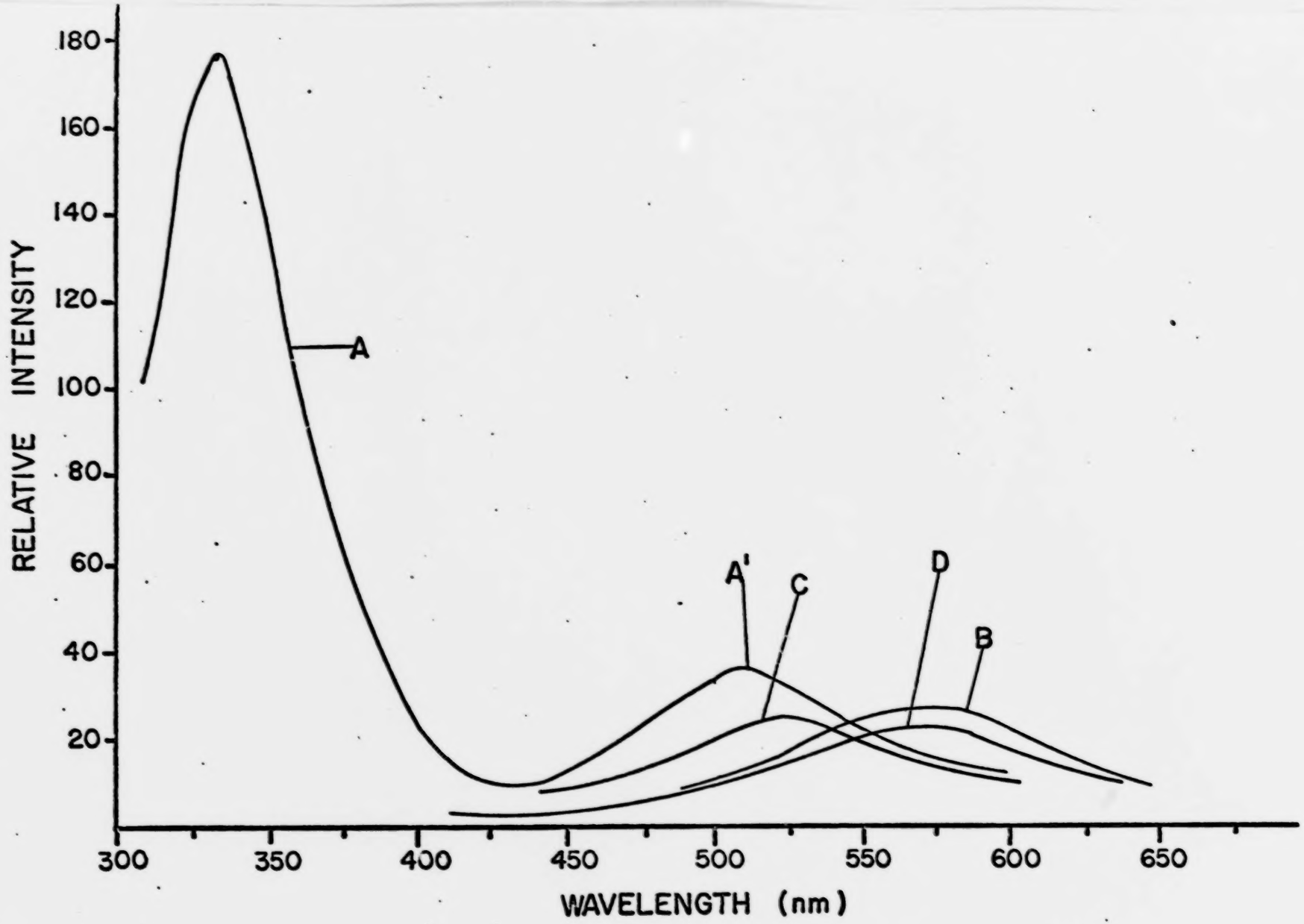


Figure II Curve A indicates the intrinsic fluorescence of L-tryptophan (340 nm) in a 0.1 mg/ml solution of horse serum cholinesterase in water. Curve A': The 515 nm emission is that of the probe enzyme complex (excitation 280 nm). Curve B represents the fluorescence response of the probe molecule in water (1×10^{-6} M), excitation 330 nm, in the presence of the enzyme (excitation at 330 nm). Curve C indicates the spectral shift of the emission of the probe-enzyme complex when DFP is added (excitation at 280 nm). Curve D represents the spectral shift which occurs in the probe emmission when DFP is added (excitation at 330 nm).

Appendix

Two publications and one draft copy of a paper in press are appended.